A secreted protein is an endogenous chemorepellant in *Dictyostelium discoideum*

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Chemorepellants may play multiple roles in physiological and pathological processes. However, few endogenous chemorepellants have been identified, and how they function is unclear. We found that the autocrine signal AprA, which is produced by growing *Dictyostelium discoideum* cells and inhibits their proliferation, also functions as a chemorepellant. Wild-type cells at the edge of a colony show directed movement outward from the colony, whereas cells lacking AprA do not. Cells show directed movement away from a source of recombinant AprA and dialyzed conditioned media from wild-type cells, but not dialyzed conditioned media from *aprA*$^-$ cells. The secreted protein CfaD, the G protein Gα8, and the kinase QkgA are necessary for the chemorepellant activity of AprA as well as its proliferation-inhibiting activity, whereas the putative transcription factor BzpN is dispensable for the chemorepellant activity of AprA but necessary for inhibition of proliferation. Phospholipase C and PI3 kinases 1 and 2, which are necessary for the activity of at least one other chemorepellant in *Dictyostelium*, are not necessary for recombinant AprA chemorepellant activity. Starved cells are not repelled by recombinant AprA, suggesting that aggregation-phase cells are not sensitive to the chemorepellant effect. Cell tracking indicates that AprA affects the directional bias of cell movement, but not cell velocity or the persistence of cell movement. Together, our data indicate that the endogenous signal AprA acts as an autocrine chemorepellant for *Dictyostelium* cells.

autocrine signaling | signal transduction | chemorepellent | gradient

Chemotaxis, or directed movement in response to a chemical gradient, is an ancient and critical behavior of eukaryotic and prokaryotic cells (1). In eukaryotes, chemotaxis proceeds through extracellular signal sensing and polarization of the actin cytoskeleton, resulting in cellular extensions (pseudopods) that facilitate movement (2). Many chemoattractants, or signals that cells move toward, have been identified (3–6), and many molecular components involved in directional sensing and regulation of actin dynamics have been characterized (7). An alternative chemotactic process involves chemorepellants, signals that cells move away from. Some chemoattractants, such as SDF-1 and interleukin-8, act as chemorepellants at high concentrations (8, 9), and the protein semaphorin III acts as a chemorepellant in the context of neuronal growth cone guidance (10). Chemorepellants may function in the resolution of inflammation (11), gastrulation (12), the pathogenicity of the parasite *Entamoeba histolytica* (13), and metastasis (14). However, few endogenous chemorepellants have been identified, and relatively little is known regarding their mechanism of action.

The eukaryote *Dictyostelium discoideum* is an excellent model for the study of chemotactic processes. In the presence of nutrients, *Dictyostelium* exist as unicellular amoebae that reproduce by fission. When starved, cells secrete and respond to the chemoattractant cAMP, leading to the aggregation of cells and the formation of fruiting bodies (15). *Dictyostelium* chemotaxis toward cAMP involves G protein-coupled receptors (16), heterotrimeric G proteins (17, 18), Ras (19, 20), PI3 kinase (PI3K) (21), phospholipases (22), and other proteins (7, 23). There also appear to be *Dictyostelium* chemorepellants. For example, when a small spot of cells is placed adjacent to a much larger spot, cells in the small spot tend to move away from the larger spot (24), and when two spots of high-density cells are placed next to each other, cells show a greater degree of movement away from the adjacent spot than toward it (25). In addition, the synthetic cAMP analog 8CPT-cAMP induces negative chemotaxis through localized phospholipase C inhibition (26). However, no endogenous chemorepellants have been identified.

AprA is an autocrine-signaling protein produced by vegetative *Dictyostelium* cells that inhibits cell proliferation (27, 28). As the local cell density increases, the concentration of AprA concomitantly increases, resulting in the inhibition of proliferation at high cell density and thus establishing a threshold for the maximum density of cells. AprA shows saturable binding to cells (27) and, although no receptor has been identified, AprA requires the heterotrimeric G protein complex components Gα8 and Gβ to inhibit proliferation, to induce high-affinity GTP binding to membranes (29), and for the GTP analog GTPγS to inhibit the binding of recombinant AprA (rAprA) to cell membranes (29). Inhibition of proliferation by AprA also requires the secreted protein CfaD (30), the ROCO kinase QkgA (31), and the putative transcription factor BzpN (32), implicating these proteins in signal transduction by AprA. Interestingly, although *aprA*$^-$ cells are able to chemotax toward cAMP, as evidenced by the ability of the cells to aggregate, and *aprA*$^-$ cells show random motility like that of wild-type cells, colonies of *aprA*$^-$ cells show a reduced rate of expansion compared with wild type (31). In this report, we provide evidence indicating that AprA functions as an autocrine chemorepellant for vegetative *Dictyostelium* cells.

Results

Wild-Type but Not *aprA*$^-$ or *cfaD*$^-$ Cells Show Directed Movement Away from Regions of High Cell Density. We previously found that colonies of *aprA*$^-$ and *cfaD*$^-$ cells expand less rapidly than wild-type cells on a lawn of bacteria, although these mutants show no difference in random cell motility at low cell density compared with wild type (31). To test whether wild-type cells show a directed movement away from areas of high cell density, we established small colonies of wild-type cells in growth media and measured the displacement of individual cells at the colony edges by video microscopy. These cells showed an average displacement away from the cell colony (Fig. 1), with 94 ± 6% of measured cells showing a displacement away from the colony (mean ± SEM, $n = 3$, at least 10 cells per experiment). For *aprA*$^-$ or *cfaD*$^-$ cells, although cells were motile, the average displacement of cells was not strongly biased in any direction, with 50 ± 9% of *aprA*$^-$ cells and 67 ± 4% of *cfaD*$^-$ cells showing displacement away from the colony. These

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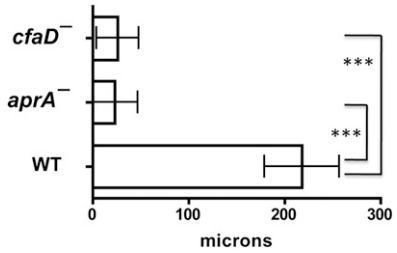

Fig. 1. Wild type, but not *aprA*$^-$ or *cfaD*$^-$ cells, show a directed movement away from a colony of cells. A spot of cells was allowed to settle in a cell culture chamber, and then the chamber was filled with media. The edge of the colony was filmed, and the movement of individual cells was followed over a period of 5 h. The average displacement of cells in the direction away from the colony is shown. Values are mean ± SEM; $n = 3$, with the displacement of at least 10 cells per genotype measured for each independent experiment. The difference between wild type and either *aprA*$^-$ or *cfaD*$^-$ is significant ($P < 0.001$, one-way ANOVA, Tukey's test).

results show that wild-type but not *aprA*$^-$ or *cfaD*$^-$ cells show a directed movement away from regions of high cell density, indicating that AprA and/or CfaD are required for directed movement away from a cell colony.

Cells Show Directed Movement Away from a Source of AprA but Not CfaD. One explanation for the directed movement of wild-type cells, but not *aprA*$^-$ or *cfaD*$^-$ cells, away from a colony is that AprA and/or CfaD may be acting as an autocrine chemorepellant, present at high concentrations where cell density is high and facilitating the spreading of cells. To test this possibility, we used an under-agarose assay (33), which consists of placing a layer of agarose over a uniform field of cells, adding rAprA or rCfaD to a well in the agarose, and examining the displacement of individual cells adjacent to the well. When medium alone was added to the well, cells adjacent to the well showed no significant bias in displacement in the direction away from the well (Fig. 2*A*), with 56 ± 4% of cells showing displacement away from the well. However, when rAprA was added to the well, cells showed an average displacement away from the well, and 73 ± 9% of cells moved away from the well. When rCfaD was added to the well, no significant bias in displacement was observed. We examined actin and myosin localization in cells in a rAprA gradient and invariably detected actin localization at the leading edge and myosin II localization at the trailing edge (Fig. S1), suggesting that cells that show directed movement away from rAprA move with the classical mechanism of actin-rich protrusions at the leading edge of the cell and myosin II-mediated contraction at the rear (2). These results indicate that AprA functions as a chemorepellant and suggest that CfaD does not share this function. Because rAprA and rCfaD were produced in identical protocols and rCfaD does not have chemorepellant activity, the chemorepellant activity of AprA is likely not due to a contaminant from protein expression and purification.

The extracellular concentration of AprA increases as a function of cell density in shaking culture and reaches a maximum concentration of 300 ng/mL (27). To test the effect of different concentrations of rAprA on cell movement, we used an Insall chamber assay (34), which is a more tractable assay for chemotaxis than the previously described under-agarose assay. We tested the response of cells to the chemoattractant folate and to rAprA and observed chemotaxis toward folate and away from rAprA (Fig. S2), showing that cells used in this assay are chemotaxis competent and chemorepulse from rAprA. A source of rAprA at a concentration of 20 ng/mL was sufficient for chemorepellant activity (Fig. 2*B*). These results indicate that the chemorepellant effect of AprA occurs at physiological concentrations.

Chemorepellant Activity Present in Dialyzed Conditioned Media from Wild-Type Cells Is Reduced in *aprA*$^-$ Conditioned Media. If AprA functions as a chemorepellant, then conditioned media from wild-type cells should have chemorepellant activity, and this activity may be reduced or absent in conditioned media from *aprA*$^-$ cells. To

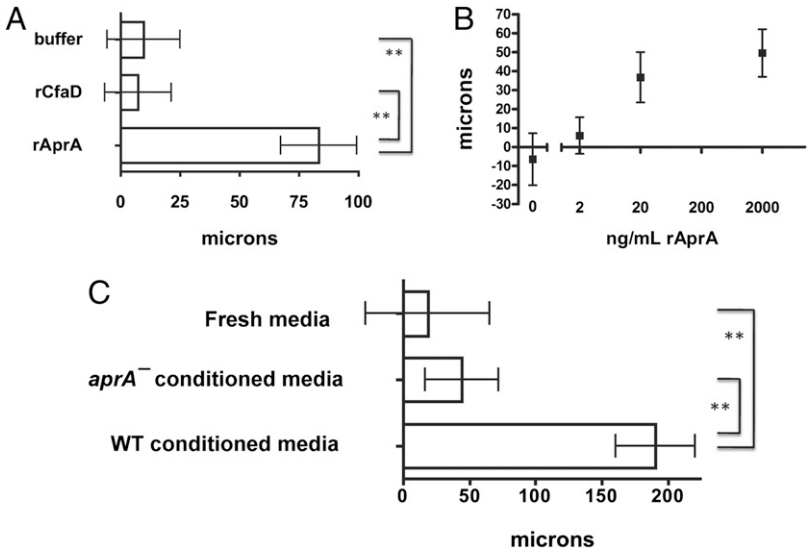

Fig. 2. rAprA but not rCfaD repels cells. (*A*) A population of wild-type cells was established under a layer of agarose, and then a well was cut in the agarose and either buffer, rCfaD, or rAprA was added. Cells adjacent to the well were filmed, and the displacement of individual cells in the direction away from the well over 2 h was measured. Values are mean ± SEM; $n \geq 4$, with the displacement of at least 10 cells per genotype measured for each independent experiment. The differences between the displacements for AprA and either buffer or CfaD are significant ($P < 0.01$, one-way ANOVA, Dunnett's test). (*B*) Wild-type cells adjacent to a source of rAprA at the indicated concentration in HL5 media were filmed for 1 h, and the displacement of individual cells away from the rAprA source was measured. The differences in average displacement are significant between either 20 or 2,000 ng/mL conditions and the condition without rAprA ($P < 0.05$, *t* test). Values are mean ± SEM; $n \geq 3$. (*C*) Under-agarose assays were done as described above using dialyzed conditioned media. Values are mean ± SEM; $n = 3$, with the displacement of at least 10 cells per genotype measured for each independent experiment. The displacements for WT conditioned media are significantly different from both fresh media and *aprA*$^-$ conditioned media ($P < 0.01$, one-way ANOVA, Tukey's test). The difference between *aprA*$^-$ conditioned media and the value 0 is not significant (paired *t* test).

test this prediction, we measured the displacement of cells adjacent to conditioned media from wild-type cells, conditioned media from *aprA*$^-$ cells, or fresh media using the under-agarose assay. Conditioned media from both wild-type and *aprA*$^-$ cells at high density had a chemorepellant effect (Fig. S3). However, when conditioned media was dialyzed against fresh media using a 10-kDa cutoff membrane and then used in the under-agarose assay, conditioned media from wild-type cells caused a significant bias in cell displacement away from the conditioned media compared with fresh media (Fig. 2*C*), whereas conditioned media from *aprA*$^-$ cells did not show a significant effect on cell displacement. These results indicate that something larger than 10 kDa that is present in wild-type conditioned media but not significantly present in *aprA*$^-$ conditioned media functions as a chemorepellant, supporting the hypothesis that AprA itself is a chemorepellant. In addition, a chemorepellant that is smaller than 10 kDa is present in high-density *aprA*$^-$ conditioned medium.

AprA Shows Chemorepellant Activity in the Absence of Rich Media. An autocrine chemorepellant could potentially work by degrading or inactivating a chemoattractant present in the surrounding area, causing the chemoattractant concentration to be low in areas of high cell density and high elsewhere. This would then create a gradient of the chemoattractant, causing cells to move away from areas of high cell density. Because at least one compound in rich media, folate, functions as a *Dictyostelium* chemoattractant (35), this model of chemorepulsion could potentially be occurring under our experimental conditions. We thus examined whether rich media is necessary for AprA chemorepellant activity by assaying for chemorepellant activity using cells in buffer. Under these conditions, we still observed a chemorepellant effect of rAprA that was not significantly different from that observed in rich media (*t* test; Fig. 3 *A* and *B*); 48 ± 5% of cells measured showed movement away from buffer (with buffer also in the opposite chamber), whereas 81 ± 4% of cells showed movement away from rAprA in buffer ($P < 0.01$, *t* test, $n = 3$). These results show that the chemorepellant effect of AprA does not require rich media, strongly suggesting that AprA does not function as a chemorepellant by inactivating a chemoattractant.

AprA Shows No Detectable Chemorepellant Activity on Starved Cells. AprA is most strongly expressed during vegetative growth, although expression is also detected during aggregation and development (28). To examine whether AprA affects the movement of cells at the aggregation stage, we starved wild-type cells for 5 h in buffer to induce the transition from growth to development and then assayed the chemorepellant effect of rAprA on these cells. Under these conditions, no bias in the direction of cell movement was evident (Fig. 3*A*). This result suggests that rAprA does not act as a chemorepellant for cells during the aggregation stage.

Chemorepellant Effect of AprA Requires CfaD, QkgA, and Gα8, but Not BzpN, Phospholipase C, or PI3 kinases 1 and 2. The G protein Gα8, the kinase QkgA, and the putative transcription factor BzpN are necessary for the proliferation-inhibiting activity of AprA (29, 31, 32), implicating these proteins in AprA signaling. Additionally, the secreted protein CfaD is necessary for AprA activity (30). To test whether these proteins are necessary for the chemorepellant activity of AprA, we examined whether cell lines mutant for these proteins were repelled by rAprA in an Insall chamber assay. We saw that wild-type cells adjacent to a source of rAprA showed a significant bias in displacement away from rAprA (Fig. 3*B* and Movies S1 and S2). Similarly, *aprA*$^-$ and *bzpN*$^-$ cells showed directed movement away from a rAprA source. In contrast, *gα8*$^-$, *qkgA*$^-$, and *cfaD*$^-$ cells showed no significant bias in displacement relative to a rAprA source. These results indicate that Gα8, QkgA, and CfaD are necessary for the chemorepellant effect of AprA, whereas BzpN is not. Consistent with our interpretation of AprA

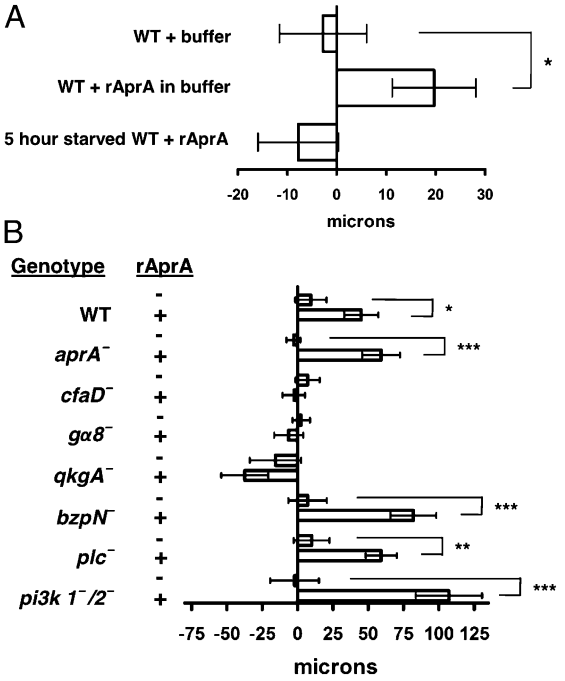

Fig. 3. AprA functions as a chemorepellant in the absence of rich media and requires the proteins Gα8, QkgA, and CfaD but not BzpN or PLC for activity. (*A*) Cells were placed adjacent to a source of rAprA or buffer using an Insall chamber and filmed for 1 h. The displacement of individual cells in the direction away from the rAprA source was then measured. Values are mean ± SEM; $n = 3$. The difference between the indicated conditions is significant ($P < 0.05$, one-tailed *t* test). The 5-h starved WT condition is not significantly different from the value 0 ($P > 0.05$, paired *t* test). (*B*) Cells in HL5 media were placed adjacent to a source of rAprA or media using an Insall chamber and filmed for 1 h. The displacement of individual cells in the direction away from the rAprA source was then measured. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (*t* test). The differences in average displacement between genotypes that show a significant chemorepellant response are not significant (one-way ANOVA, Tukey's test).

chemorepellant activity acting to disperse cell colonies, we found that, like *aprA*$^-$, *cfaD*$^-$, and *qkgA*$^-$ cells, *gα8*$^-$ cells showed a reduced spreading at the edge of cell colonies compared with wild type, whereas *bzpN*$^-$ cells did not (Fig. S4).

The synthetic cAMP analog 8CPT-cAMP acts as a chemorepellant for starved *Dictyostelium* cells, and this chemorepulsion requires phospholipase C (PLC) and the activity of PI3 kinases 1 and 2 (26). We observed that both *plc*$^-$ and *pi3k1*$^-$*/2*$^-$ cells showed movement away from a rAprA source in a manner similar to wild-type cells (Fig. 3*B*). We further examined the role of PI3K activity in AprA-mediated chemorepulsion by using cells expressing cytosolic regulator of adenylate cyclase (CRAC)-GFP, a marker for PI3K activity at the cell cortex, which localizes to the upgradient side of a cell during chemotaxis toward cAMP (36). When situated in a gradient of rAprA, cell tracking over a 5-min period revealed that 54% of cells expressing CRAC-GFP moved down the rAprA gradient, 27% of cells moved up the gradient, and 19% of cells moved roughly parallel to the gradient, suggesting that rAprA has a chemorepellant effect on these cells. When CRAC-GFP was imaged, the majority of cells showed cytosolic CRAC-GFP localization, and localization did not correlate with the direction of the rAprA gradient for the whole-cell population or for the subset of cells moving down the rAprA gradient (Fig. S5). This result indicates that PLC and PI3K activity are not necessary for the chemorepellant activity of AprA and that AprA and 8CPT-cAMP function as chemorepellants through distinct mechanisms.

AprA Biases the Direction of Cell Movement but Does Not Affect Average Cell Speed. To gain insight into the mechanism by which AprA might affect cell movement, we tracked the movement of wild-type cells in a gradient of rAprA over 1 h and examined the parameters of cell movement. Whereas no bias in the direction of movement was evident for a media control (Fig. 4*A*), cells tended to move away from the rAprA source, and the center of mass of the cell endpoints indicated a bias away from the rAprA source (Fig. 4*B*). Tracks of cells in a gradient of rAprA showed a negative forward migration index (FMI) that was significantly different from the FMI for the media control (Table 1), supporting the interpretation of AprA as a chemorepellant. The average speed of cells in a gradient of rAprA was not significantly different from the control (Table 1) and was consistent with previous measurements of randomly motile vegetative cells (37, 38), indicating that AprA does not stimulate an increase in the average speed of cells. Supporting this conclusion, cells in a uniform concentration of 2 μg/mL rAprA showed a displacement of 152 ± 33 μm over an interval of 2 h, whereas cells in media showed a displacement of 157 ± 19 μm; the difference is not significant. Cells in a gradient of rAprA showed a significantly higher directionality (a measure of how directed, as opposed to random, the movement of a cell is) than the control (Table 1), further indicating that AprA affects the direction of cell movement. Finally, we examined whether the distribution of the endpoints of cell tracks was nonrandom by performing a Rayleigh test. The distribution for cell endpoints adjacent to a rAprA source was significantly biased, whereas the distribution of endpoints for the media control was not (Table 1). Together, these data indicate that AprA regulates the directionality of cell movement so that cells move away from high AprA concentrations and that this regulation does not involve a change in average cell speed.

AprA Affects Directionality but Not Persistence of Cell Movement. To further characterize how AprA affects movement, we used the cell-tracking data to examine how AprA might affect the directional persistence of cells. Movies of cells were processed into 35-s intervals, and the displacement of cells in the direction of the AprA source was determined for all cells and all intervals. Most commonly, cells showed no change in position over a 35-s interval (Fig. S6). For the control condition, the distribution of displacements revealed no bias in movement, whereas for cells in a gradient of rAprA a bias in displacement in the direction away from rAprA was evident (Fig. S6 and Table 2). We then examined the average displacement in all intervals in the direction toward or away from the rAprA source to determine whether rAprA might cause an increase in cell velocity in certain directions. There was no significant difference in the average displacement for cells moving away from the source between the rAprA and the control condition, and there was no significant difference in the average displacement for cells moving toward the source between the two conditions (Table 2). These results suggest that AprA does not significantly increase the velocity of cells in a directional manner.

To determine the effect of rAprA on the persistence of cell movement, we first defined P_A as the probability that a cell would move away from rAprA, and P_T as the probability that a cell would move toward rAprA. As shown in Table 2, P_A and P_T are roughly the same for the media control and significantly different in a gradient of rAprA. We then measured, for each cell, P_{A2}, the percentage of intervals where the cell moved away from rAprA in one interval and away again in the next interval. Because the probability that a cell will move away is different depending on whether there is a gradient of rAprA, we calculated NA2 = $P_{A2}/(P_A)^2$, or the observed probability of two subsequent movements away from rAprA divided by the expected probability that such an event would happen at random. NA2 thus represents a normalized probability that a cell will move away from rAprA in two subsequent time intervals, with higher values indicating more persistence in movement than would be expected at random. We found that NA2 was not statistically different between the rAprA gradient condition and the control and that the corresponding NT2 (the normalized probability of a cell moving toward rAprA in two sequential 35-s intervals) was also not different between the rAprA gradient and control (Table 2). We similarly calculated the normalized probability that a cell will move away from rAprA in either three or four consecutive intervals as NA3 = $P_{A3}/(P_A)^3$ or NA4 = $P_{A4}/(P_A)^4$. For both control and rAprA gradient conditions, NA4 was greater than NA3, and NA3 was greater than NA2, suggesting that longer runs of persistence occur at a higher rate than would be expected at random compared with shorter ones. We observed no statistically significant difference in the normalized probability that a cell moved away in three or four consecutive time intervals between rAprA gradient and control conditions. Similarly, the corresponding NT3 and NT4 values are not significantly different between rAprA gradient and control experiments. Together, these results suggest that the chemorepulsion mediated by rAprA is not due to an altered persistence in movement away from or toward the source of rAprA.

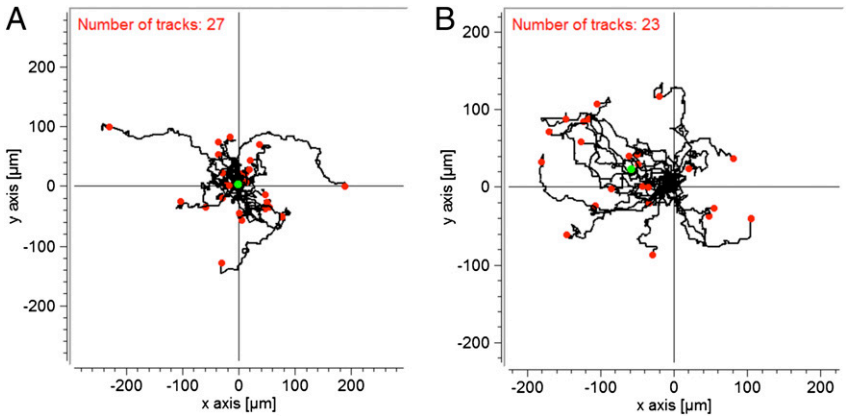

Fig. 4. Tracking of cells adjacent to an AprA source. Wild-type cells in the absence (*A*) or presence (*B*) of a rAprA gradient were filmed using an Insall chamber; cells were tracked over a 1-h period, and tracks were graphed. The AprA source is on the right side of the origin. Red dots represent the final position of cells, and green dots are the averaged center of mass for all tracks. The tracks are a compilation of three independent experiments with at least seven tracks per experiment.

Table 1. Effect of AprA on FMI, cell speed, cell directionality, and cell distribution

	Media control	rAprA
FMI	−0.01 ± 0.04	−0.18 ± 0.06*
Speed (μm/min)	4.9 ± 0.2	5.0 ± 0.2
Directionality	0.21 ± 0.03	0.34 ± 0.03***
Rayleigh test (*P* value)	0.52	0.003

The data from Fig. 4 were analyzed to calculate the forward migration index, or FMI (a measure of cell movement in the direction of a gradient in respect to total cell movement, with zero indicating no movement in the direction of the gradient), cell speed, directionality (the ratio of the Euclidean distance to the total distance traveled), and *P* values for the Rayleigh test, a test for nonrandom distributions of cell endpoints. *$P < 0.05$ indicates a significant difference; ***$P < 0.001$ (*t* test).

Discussion

Colonies of *aprA*$^-$ cells expand less rapidly than wild-type colonies, despite the fact that *aprA*$^-$ cells proliferate more rapidly than wild-type cells (28, 31). Our data support the hypothesis that rAprA functions as an autocrine chemorepellant for *Dictyostelium* cells and that this chemorepellant function may facilitate the spreading of cell colonies.

We found that dialyzed conditioned media from wild-type but not *aprA*$^-$ cells has chemorepellant activity. However, when conditioned media was taken directly from cells, filter-sterilized, and assayed, conditioned media from *aprA*$^-$ cells showed chemorepellant activity. This result suggests that a small molecule or molecules that have chemorepellant activity accumulate in the media of high-density cultures of *aprA*$^-$ cells. Alternatively, conditioned media from high-density cells may be depleted of nutrients, and using this depleted media could establish a gradient of nutrients leading away from the source, which might lead to cells moving away. Regardless, this chemorepellant effect is likely not functioning physiologically in the movement of cells away from areas of high cell density, as *aprA*$^-$ cells do not show a strong bias in movement away from a colony (Fig. 1).

Multiple lines of evidence suggest that AprA acts as a ligand for a G protein-coupled cell-surface receptor as opposed to interacting with another extracellular factor or factors (27, 29). We found that rAprA shows chemorepellant activity in the absence of rich media, indicating that AprA does not function by modifying factors in media with chemokinetic properties. This result further suggests that AprA acts as a ligand as opposed to modifying an extracellular factor.

We found that the G protein Gα8 and the kinase QkgA are necessary for the chemorepellant activity of AprA as well as for its proliferation-inhibiting activity, but that that the putative transcription factor BzpN is dispensable for AprA chemorepellant activity despite being necessary for inhibition of proliferation. These results suggest that AprA inhibits proliferation and induces chemorepulsion using partially overlapping signal transduction pathways and that the signal transduction branches, with BzpN being a component of the proliferation-inhibiting pathway but not the chemorepellant pathway.

Whereas the previously reported *Dictyostelium* chemorepellant 8CPT-cAMP requires PLC and PI3 kinases 1 and 2 for activity, we found that these proteins are not required for the chemorepellant activity of rAprA. Furthermore, whereas 8CPT-cAMP acts on starved cells (26), rAprA shows no chemorepellant activity on starved cells. These results suggest that these chemorepellants likely function through different mechanisms and that, when cells are aggregating, the AprA chemorepulsion mechanism is disabled. We further investigated the role of PI3-kinase activity on chemorepulsion and saw that CRAC-GFP localization did not correspond to a rAprA gradient, suggesting that polarized PI3-kinase activity is not essential for rAprA chemorepellant activity.

We tracked the movement of cells in a gradient of rAprA and found that cells showed a negative migration index and more directed movement than control cells, although the difference in average speed for cells in a rAprA gradient and control cells was not significant. We also found that AprA does not affect the persistence of cell movement in a directional manner. Taken together, our data indicate that rAprA functions as a chemorepellant by biasing the movement of cells in the direction of a decreasing AprA gradient, and not by affecting the speed of cells or the persistence of cell movement.

A *Dictyostelium* autocrine chemorepellant mechanism may have been selected for because it spreads out groups of vegetative cells, as cells would tend to move away from areas of high cell density by moving down an AprA gradient. This movement would result in coverage of a larger area by a population of cells and thus provide access to a larger quantity of nutrients, facilitating growth. Such an effect seems evident during growth of wild-type or *aprA*$^-$ cells on lawns of bacteria, as wild-type colonies expand and clear the bacterial lawn more rapidly than *aprA*$^-$ cell colonies (31). The proliferation-inhibiting activity of AprA (28) may act in cooperation

Table 2. Effect of AprA on the direction of cell displacement and cell persistence over 35-s intervals

		Media control	rAprA
Probabilities of cell displacement over one 35-s interval	Displacement away from source (P_A)	0.27 ± 0.02	0.35 ± 0.01**
	Displacement toward source (P_T)	0.28 ± 0.02	0.20 ± 0.02*
	No displacement	0.46 ± 0.01	0.44 ± 0.01
Average displacement of indicated subset (μm)	Displacements away from source	3.3 ± 0.1	3.4 ± 0.1
	Displacements toward source	3.1 ± 0.1	3.1 ± 0.1
Normalized probability of two subsequent movements in the same direction	Away from source (NA2)	1.3 ± 0.2	1.4 ± 0.1
	Toward source (NT2)	1.4 ± 0.1	1.7 ± 0.2
Normalized probability of three subsequent movements in the same direction	Away from source (NA3)	2.0 ± 0.7	2.0 ± 0.1
	Toward source (NT3)	2.1 ± 0.1	2.8 ± 0.7
Normalized probability of four subsequent movements in the same direction	Away from source (NA4)	3.7 ± 1.7	2.8 ± 0.1
	Toward source (NT4)	4.8 ± 0.6	5.5 ± 2.1

The data from cell tracking are shown here as the probability that, in a 35-s interval, a cell will show displacement away from the rAprA source, toward the source, or no displacement. Values are mean ± SEM from three independent experiments. *$P < 0.05$ indicates that the differences between control and rAprA gradient conditions are significant; **$P < 0.01$ (*t* test). For the control, P_A and P_T are not significantly different, whereas in a gradient of rAprA, P_A and P_T are significantly different with $P < 0.01$ (*t* test). For all normalized probabilities of subsequent movements in the same direction, differences between the control and rAprA conditions are not significant ($P > 0.05$, *t* test).

with this process by preventing proliferation at high cell density, and thus conserving local resources, giving high-density cells the opportunity to spread before being depleted of nutrients and undergoing aggregation.

Few endogenous chemorepellants have been identified, and their mechanisms of action are largely unknown. We have identified an endogenous chemorepellant in *D. discoideum* and have identified conserved proteins necessary for chemorepellant function that may serve as components of a signal transduction pathway. *Dictyostelium*, a highly tractable model organism, has been immensely useful in elucidating conserved chemotactic mechanisms. This fact suggests the exciting possibility that the study of AprA and its mechanism of action could shed light on conserved mechanisms of chemorepulsion, perhaps providing insight or therapeutic approaches for disease states in which chemorepellants play a role.

Materials and Methods

Cell Culture and Recombinant Protein Purification. The strains Ax2 (wild type), *aprA*$^-$ [DBS0235509 (28)], *cfaD*$^-$ [DBS0302444 (30)], *qkgA*$^-$ [DBS0236839 (39)], *bzpN*$^-$ (32), *gα8*$^-$ [DBS0236107 (40)], *plc*$^-$ [DBS0236793 (26)], *pi3k1*$^-$*/2*$^-$ [DBS0236766 (41)], and Ax2/CRAC-GFP [DBS0235626 (36)] were grown in axenic shaking culture as described previously (42) except that Formedium HL5 media was used (Formedium). Recombinant AprA and CfaD were expressed and purified from *Escherichia coli* as described previously (28, 30).

Insall Chamber Assay. To measure the effect of AprA on cell displacement using an Insall chamber (34), cells in shaking culture were diluted to 5×10^4 cells/mL in HL5 media, and then 300-μL volumes of the dilution were grown on 22- × 22-mm glass coverslips for 1 h at 20 °C. We then used an Insall chamber slide, a kind gift from Robert Insall (Beatson Institute for Cancer Research, Glasgow, UK), consisting of two concentric square depressions separated by a bridge (34). Both depressions and the bridge were filled with HL5 media, and then the media was removed from the coverslips, which were then placed face down on the chamber. Media was then removed from the outer chamber and was replaced by either rAprA in HL5 or HL5 alone. Cells located on the bridge between the square depressions were then filmed using a 10× objective. After an initial 20-min period, the displacement of at least 10 individual cells per experiment was measured over a period of 1 h. The displacement of cells in the direction away from the rAprA source was then calculated by vector decomposition. To analyze cell displacement in the absence of rich media, cells were grown on coverslips for 1 h as described above, then media was removed, and cells were washed twice with 300 μL PBM [20 mM KH_2PO_4, 0.01 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 6.1) with KOH]. The Insall chamber assay was then performed as described above, except that PBM was used in place of HL5 for all steps.

For measurement of cell displacement at colony edges; under-agarose assays; measurement of cell movement in uniform rAprA concentrations; cell tracking; imaging of GFP fusion protein localization in live cells; and statistics, see *SI Materials and Methods*.

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1. Porter SL, Wadhams GH, Armitage JP (2011) Signal processing in complex chemotaxis pathways. *Nat Rev Microbiol* 9(3):153–165.
2. Rappel WJ, Loomis WF (2009) Eukaryotic chemotaxis. *Wiley Interdiscip Rev Syst Biol Med* 1(1):141–149.
3. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 184:1101–1109.
4. Ward PA (1967) A plasmin-split fragment of C′3 as a new chemotactic factor. *J Exp Med* 126:189–206.
5. Barkley DS (1969) Adenosine-3′,5′-phosphate: Identification as acrasin in a species of cellular slime mold. *Science* 165:1133–1134.
6. Yoshimura T, et al. (1989) Three forms of monocyte-derived neutrophil chemotactic factor (MDNCF) distinguished by different lengths of the amino-terminal sequence. *Mol Immunol* 26(1):87–93.
7. Franca-Koh J, Kamimura Y, Devreotes P (2006) Navigating signaling networks: Chemotaxis in Dictyostelium discoideum. *Curr Opin Genet Dev* 16:333–338.
8. Poznansky MC, et al. (2000) Active movement of T cells away from a chemokine. *Nat Med* 6:543–548.
9. Tharp WG, et al. (2006) Neutrophil chemorepulsion in defined interleukin-8 gradients in vitro and in vivo. *J Leukoc Biol* 79:539–554.
10. Messersmith EK, et al. (1995) Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* 14:949–959.
11. Vianello F, Olszak IT, Poznansky MC (2005) Fugetaxis: Active movement of leukocytes away from a chemokinetic agent. *J Mol Med (Berl)* 83:752–763.
12. Yang X, Dormann D, Münsterberg AE, Weijer CJ (2002) Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. *Dev Cell* 3:425–437.
13. Zaki M, Andrew N, Insall RH (2006) Entamoeba histolytica cell movement: A central role for self-generated chemokines and chemorepellents. *Proc Natl Acad Sci USA* 103: 18751–18756.
14. Bagci T, Wu JK, Pfannl R, Ilag LL, Jay DG (2009) Autocrine semaphorin 3A signaling promotes glioblastoma dispersal. *Oncogene* 28:3537–3550.
15. Kessin RH (2001) *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge Univ. Press, Cambridge, UK), p xiv, 294 p.
16. Klein PS, et al. (1988) A chemoattractant receptor controls development in Dictyostelium discoideum. *Science* 241:1467–1472.
17. Kumagai A, et al. (1989) Regulation and function of G alpha protein subunits in Dictyostelium. *Cell* 57:265–275.
18. Brzostowski JA, Parent CA, Kimmel AR (2004) A G alpha-dependent pathway that antagonizes multiple chemoattractant responses that regulate directional cell movement. *Genes Dev* 18:805–815.
19. Reymond CD, et al. (1986) Phenotypic changes induced by a mutated ras gene during the development of Dictyostelium transformants. *Nature* 323:340–343.
20. Chubb JR, Wilkins A, Thomas GM, Insall RH (2000) The Dictyostelium RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *J Cell Sci* 113:709–719.
21. Funamoto S, Milan K, Meili R, Firtel RA (2001) Role of phosphatidylinositol 3′ kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in dictyostelium. *J Cell Biol* 153:795–810.
22. Bominaar AA, Van Haastert PJ (1993) Chemotactic antagonists of cAMP inhibit Dictyostelium phospholipase C. *J Cell Sci* 104(Pt 1):181–185.
23. Booth EO, Van Driessche N, Zhuchenko O, Kuspa A, Shaulsky G (2005) Microarray phenotyping in Dictyostelium reveals a regulon of chemotaxis genes. *Bioinformatics* 21:4371–4377.
24. Kakebeeke PI, de Wit RJ, Kohtz SD, Konijn TM (1979) Negative chemotaxis in Dictyostelium and Polysphondylium. *Exp Cell Res* 124:429–433.
25. Keating MT, Bonner JT (1977) Negative chemotaxis in cellular slime molds. *J Bacteriol* 130(1):144–147.
26. Keizer-Gunnink I, Kortholt A, Van Haastert PJ (2007) Chemoattractants and chemorepellents act by inducing opposite polarity in phospholipase C and PI3-kinase signaling. *J Cell Biol* 177:579–585.
27. Choe JM, Bakthavatsalam D, Phillips JE, Gomer RH (2009) Dictyostelium cells bind a secreted autocrine factor that represses cell proliferation. *BMC Biochem* 10:4.
28. Brock DA, Gomer RH (2005) A secreted factor represses cell proliferation in Dictyostelium. *Development* 132:4553–4562.
29. Bakthavatsalam D, Choe JM, Hanson NE, Gomer RH (2009) A Dictyostelium chalone uses G proteins to regulate proliferation. *BMC Biol* 7:44.
30. Bakthavatsalam D, et al. (2008) The secreted Dictyostelium protein CfaD is a chalone. *J Cell Sci* 121:2473–2480.
31. Phillips JE, Gomer RH (2010) The ROCO kinase QkgA is necessary for proliferation inhibition by autocrine signals in Dictyostelium discoideum. *Eukaryot Cell* 9: 1557–1565.
32. Phillips JE, Huang E, Shaulsky G, Gomer RH (2011) The putative bZIP transcription factor BzpN slows proliferation and functions in the regulation of cell density by autocrine signals in Dictyostelium. *PLoS ONE* 6:e21765.
33. Woznica D, Knecht DA (2006) Under-agarose chemotaxis of Dictyostelium discoideum. *Methods Mol Biol* 346:311–325.
34. Muinonen-Martin AJ, Veltman DM, Kalna G, Insall RH (2010) An improved chamber for direct visualisation of chemotaxis. *PLoS ONE* 5:e15309.
35. Pan P, Hall EM, Bonner JT (1972) Folic acid as second chemotactic substance in the cellular slime moulds. *Nat New Biol* 237(75):181–182.
36. Parent CA, Blacklock BJ, Froehlich WM, Murphy DB, Devreotes PN (1998) G protein signaling events are activated at the leading edge of chemotactic cells. *Cell* 95(1): 81–91.
37. Tuxworth RI, et al. (1997) Dictyostelium RasG is required for normal motility and cytokinesis, but not growth. *J Cell Biol* 138:605–614.
38. Hoeller O, Kay RR (2007) Chemotaxis in the absence of PIP3 gradients. *Curr Biol* 17: 813–817.
39. Abe T, Langenick J, Williams JG (2003) Rapid generation of gene disruption constructs by in vitro transposition and identification of a Dictyostelium protein kinase that regulates its rate of growth and development. *Nucleic Acids Res* 31:e107.
40. Wu L, Gaskins C, Zhou K, Firtel RA, Devreotes PN (1994) Cloning and targeted mutations of G alpha 7 and G alpha 8, two developmentally regulated G protein alpha-subunit genes in Dictyostelium. *Mol Biol Cell* 5:691–702.
41. Chung CY, Potikyan G, Firtel RA (2001) Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. *Mol Cell* 7:937–947.
42. Brock DA, Gomer RH (1999) A cell-counting factor regulating structure size in Dictyostelium. *Genes Dev* 13:1960–1969.

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